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Effects of CO_2 Concentration during Growth on Fatty Acid Composition in Microalgae[1]

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ABSTRACT

The degree of unsaturation of fatty acids was higher in *Chlorella vulgaris* 11h cells grown with air (low-CO_2 cells) than in the cells grown with air enriched with 2% CO_2 (high-CO_2 cells). The change in the ratio of linoleic acid to α-linolenic acid was particularly significant. This change of the ratio was observed in four major lipids (monogalactosyldiacylglycerol, digalactosyldiacylglycerol, phosphatidylcholine, and phosphatidylethanolamine). The relative contents of lipid classes were essentially the same both in high-CO_2 and low-CO_2 cells. After high-CO_2 cells were transferred to low CO_2 condition, total amount of fatty acids remained constant but the relative content of α-linolenic acid increased during a 6-hour lag phase in growth with concomitant decreases in linoleic and oleic acids. When low-CO_2 cells were transferred to high CO_2 condition, total amount of fatty acids and relative content of oleic acid increased significantly. The amount of α-linolenic acid remained almost constant, while the amounts of palmitic, oleic, and linoleic acids increased. Similar, but smaller, changes in fatty acid compositions were observed in two species of green algae *Chlamydomonas reinhardtii* and *Dunaliella tertiolecta*. However, no difference was found in *Euglena gracilis*, *Porphyridium cruentum*, *Anabaena variabilis*, and *Anacystis nidulans*.

The affinity for inorganic carbon in photosynthesis of microalgae as well as submersed angiosperm is reduced when the concentration of CO_2 is elevated to 1 to 5% (*e.g.* refs. 3 and 19). It is generally assumed that this is due to decreases in the activity of carbonic anhydrase and in the capacity of accumulation of inorganic carbon in high-CO_2 cells.[2] Likewise the development of pyrenoids by some green algae (12, 18) and carboxysome by cyanobacteria (20) has been reported in low-CO_2 cells. From electron microscopic examination the chloroplast envelope is electronically denser in low-CO_2 cells than in high-CO_2 cells, while the opposite effect of CO_2 was observed for the plasma membrane of *Dunaliella tertiolecta* (18). Thus, CO_2 concentration during growth gives various effects on microalgae. Although protein composition and starch components have been studied so far, CO_2-dependent changes in lipid composition has not been investigated.

In the present study, we have investigated the lipid and fatty acid compositions in low-CO_2 and high-CO_2 cells as well as the variations in fatty acid composition after shifting the CO_2-concentration downward or upward during the algal growth.

MATERIALS AND METHODS

Algal Culture

The algal strains used were *Chlorella vulgaris* 11h (Algensammulung der Pflanzenphysiologischen Instituts der Unverität Göttingen), *Dunaliella tertiolectra* (from Dr. R. McC. Lilley of the University of Wollongong, Australia), *Anacystis nidulans* R2 (from Dr. K. Shinozaki of Nagoya University). *Chlamydomonas reinhardtii* (C-9), *Euglena gracilis* Krebs strain Z Pringsheim (E-6), *Porphyridium cruentum* (R-1), and *Anabaena variabilis* (M-3) were from Microbial and Microalgal Research Center, Institute of Applied Microbiology, University of Tokyo.

Cells were grown axenically in an oblong glass vessel under constant illumination with a bank of fluorescent lamps (15–20 W m^{-2}) at 28 to 30 °C except *E. gracilis* which was cultured at 25 °C. Cell suspension was bubbled with ordinary air to obtain low-CO_2 cells or air enriched with 2% CO_2 to obtain high-CO_2 cells. Culture media for the algal species were as follows: *C. vulgaris* 11h, as described by Hogetsu and Miyachi (9); *C. reinhardtii*, 3/10 HSM medium (16); *D. tertiolecta* and *P. cruentum*, as described by Aizawa and Miyachi (2); *E. gracilis* Z, by Suzuki *et al.* (17); *A. variabilis*, by Abe *et al.* (1); *A. nidulans*, by Allen (4). Cells were harvested at the late logarithmic or linear growth phase unless otherwise mentioned and were collected by centrifugation. The pcv was determined by centrifugation of the algal suspension in a hematocrit at 1500*g* for 15 min. The cells were kept frozen at −80 °C until the analyses of lipids and fatty acids.

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[2] Abbreviations: high-CO_2 cells, cells grown with air enriched with 2% CO_2; low-CO_2 cells, cells grown with ordinary air; pcv, packed cell volume; 14:0, myristic acid; 14:1, myristoleic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 16:2, hexadecadienoic acid; 16:3, hexadecatrienoic acid; 16:4, hexadecatetraenoic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, α-linolenic acid; 18:3 (*n*-6), γ-linolenic acid; 20:2, icosadienoic acid; 20:4, arachidonic acid; 20:5, icosapentaenoic acid; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

Table I. *Glycolipid and Phospholipid Composition of Chlorella vulgaris 11h Grown with Air or Air Enriched with CO_2*

Lipid	Percent of Total	
	High-CO_2 cells	Low-CO_2 cells
MGDG	34.7	35.2
DGDG	28.5	29.2
PC	20.1	17.7
PE	11.8	11.3
Others[a]	4.9	6.0

[a] Others include sulfoquinovosyldiacylglycerol, phosphatidylglycerol, and phosphatidylinositol. Total amounts of fatty acids (μmol per g dry cells) were as follows: 173 ± 22 (high-CO_2 cells) and 221 ± 19 (low-CO_2 cells).

Extraction of Lipids

The total lipids were extracted according to Bligh and Dyer (6). The lipids were fractionated by two-dimensional TLC on silica gel (Merck precoated plates, 5721). The first solvent system was chloroform:methanol:water (130:50:8, v/v/v) and the second was acetone:benzene:methanol:water (8:3:2:1, v/v/v/v). Lipid components were identified by comparing R_F values with known standards. The spots of lipids were visualized by spraying the primulin reagent (22). Silica gel of the lipid zones was scrapped off and the lipids absorbed to the silica gel were directly transmethylated with 5% anhydrous methanolic HCl as described below.

Analysis of Fatty Acid Composition

Fatty acid methyl esters were prepared from the extracted lipids or the lyophilized cells by transmethylation with 5% anhydrous methanolic HCl and analyzed by capillary gas-liquid chromatography with a Shimadzu GC-14A gas chromatograph equipped with a hydrogen flame-ionization detector (5). They were applied to a Shimadzu ULBON HR-Thermon 3000A capillary column (0.32 mm × 25 m) at 180 °C. Fatty acid methyl esters were identified by comparing the retention time with known standards and also by mass spectroscopy. The quantities of fatty acids were estimated from the peak area on the chromatogram using docosanoic acid as an internal standard. Relative contents of glycolipids and phospholipids were estimated from the amounts of fatty acids of each lipid class.

RESULTS

Effect of CO_2 Concentration on Fatty Acid Compositions of Lipids from *C. vulgaris* 11h

Analysis of the total lipids by the thin-layer chromatography showed that MGDG, DGDG, PC, and PE were major lipid components in *C. vulgaris* 11h. There were also sulfoquinovosyldiacylglycerol, phosphatidylglycerol and phosphatidylinositol as minor components. No significant difference was found in the relative contents of lipid classes between high-CO_2 and low-CO_2 cells (Table I).

Major fatty acids in *C. vulgaris* 11h were palmitic (16:0), linoleic (18:2) and α-linolenic (18:3) acids. Myristic (14:0), palmitoleic (16:1), hexadecadienoic (16:2), hexadecatrienoic (16:3), stearic (18:0) and oleic (18:1) acids were also found as minor components (Table II). Remarkable changes associated with CO_2 concentration were observed in 18:2 and 18:3. In high-CO_2 cells, the relative content of 18:2 was higher, while that of 18:3 was much lower than in low-CO_2 cells. As a result, the degree of unsaturation decreased upon increasing CO_2 concentration from 0.04% to 2%. In spite of these changes in unsaturation, the relative contents of C_{16} and C_{18} acids remained almost unchanged. Table III shows fatty acid compositions of four major lipids extracted from high-CO_2 and low-CO_2 cells. Three major fatty acids were found in DGDG, PC, and PE. In MGDG, content of 16:0 was low and relatively high contents of 16:2 and 16:3 were noted. The ratio of 18:2/18:3 in four major lipids was different between high-CO_2 and low-CO_2 cells. However, the relative differences were markedly greater in galactolipids than in the phospholipids. Since MGDG and DGDG are known as chloroplastic lipids, fatty acid compositional changes associated with CO_2 concentration might mainly reflect changes in thylakoid membranes.

The rate of growth under high CO_2 condition is higher than that under low CO_2 condition in the linear phase. Also, aeration with 2% CO_2 caused a downward shift of pH by about 0.3 units in the culture medium. Moreover, pH in the culture medium increases gradually with cell growth due to the consumption of NO_3^-. Therefore, the changes in fatty acid composition were also followed during the course of growth under either ordinary air or air enriched with 2% CO_2. It was found that the fatty acid composition was fairly constant in the linear growth phase whenever the concentration of cells was kept in the range from 0.5 to 6 ml pcv/L. These results suggested that alterations in the composition of the fatty acids (Table II) were actually dependent on the CO_2 concentration during growth. In the following CO_2-shift experiments, the cellular concentration was maintained within this range of 0.5 to 6 ml pcv/L.

Table II. *Fatty Acid Composition of Total Lipids from Chlorella vulgaris 11h Grown with Air or Air Enriched with CO_2*

Fatty Acid	Fatty Acid Composition	
	High-CO_2 cells	Low-CO_2 cells
	mol%	
14:0	3.0	3.2
16:0	24.5	23.8
16:1	0.8	0.8
16:2	5.8	2.4
16:3	1.3	7.4
18:0	1.8	1.0
18:1	5.1	1.0
18:2	39.8	22.2
18:3	17.8	38.1
C_{14} acids	3.0	3.2
C_{16} acids	32.4	34.4
C_{18} acids	64.5	62.3
18:2/18:3	2.24	0.58

Table III. *Effect of CO_2 Concentration on Fatty Acid Compositions of Major Lipids*

Fatty Acid	Fatty Acid Composition							
	MGDG		DGDG		PC		PE	
	High-CO_2 cells	Low-CO_2 cells	High-CO_2 cells	Low-CO_2 cells	High-CO_2 cells	Low-CO_2 cells	High-CO_2 cells	Low-CO_2 cells
				mol%				
14:0	1.0	ND[a]	1.9	1.4	ND	1.7	2.9	ND
16:0	3.1	1.6	12.8	12.8	34.5	38.5	45.5	41.6
16:1	2.3	ND	1.5	ND	ND	4.3	5.0	ND
16:2	14.1	9.6	4.8	2.0	ND	ND	ND	ND
16:3	7.2	12.4	0.8	0.9	ND	ND	ND	ND
18:0	1.2	1.7	1.0	0.5	2.4	2.6	4.0	0.7
18:1	1.3	ND	2.3	ND	4.5	3.5	9.6	1.5
18:2	37.1	7.6	49.8	19.6	48.7	28.6	29.4	35.2
18:3	32.6	67.1	25.1	62.7	9.9	20.8	3.7	21.0
18:2/18:3	1.13	0.11	1.98	0.31	4.92	1.38	7.95	1.68

[a] ND = not detected.

Changes in Fatty Acid Composition of *C. vulgaris* 11h upon Shifting from 2% CO_2 to 0.04%

When high-CO_2 cells were transferred to low-CO_2 condition, the relative content of 18:3 increased and 18:1 and 18:2 decreased significantly in 12 h (Fig. 1). Thereafter the content of these C_{18} acids gradually changed and after 48 h the level of each was very close to that in low-CO_2 cells (data not shown). In contrast to the pronounced changes among C_{18} acids, the variation among the C_{16} acids were small and slow. The total content of C_{16} and C_{18} acids were constant during the experimental period.

The amounts of major fatty acids per unit dry weight of cells and the pcv in a unit volume of culture were determined (data not shown), when high-CO_2 cells were transferred to ordinary air. The amounts of total fatty acids and 16:0 per unit weight of cells remained almost constant during the 24 h period. There was a lag period in growth for the first 6 h after the transfer. During this lag a major change in the amounts of 18:1, 18:2, and 18:3 occurred. The reciprocal relationships between the decreases in 18:1 and 18:2 and the increase in 18:3 suggest that the pre-existing 18:1 and 18:2 might be desaturated to 18:3 without *de novo* synthesis of fatty acids.

Changes in Fatty Acid Composition of *C. vulgaris* 11h upon Shifting from 0.04% CO_2 to 2%

The transfer of low-CO_2 cells to high CO_2 condition caused changes in both the C_{16} and C_{18} fatty acid composition (Fig. 2). The percentage of total C_{16} acids decreased and that of C_{18} acids increased. The greatest increase was observed in 18:1. The relative contents of 18:1 and 18:2 increased, while those of 16:0 and 18:3 decreased.

Figure 3 shows changes in the amounts of fatty acids per

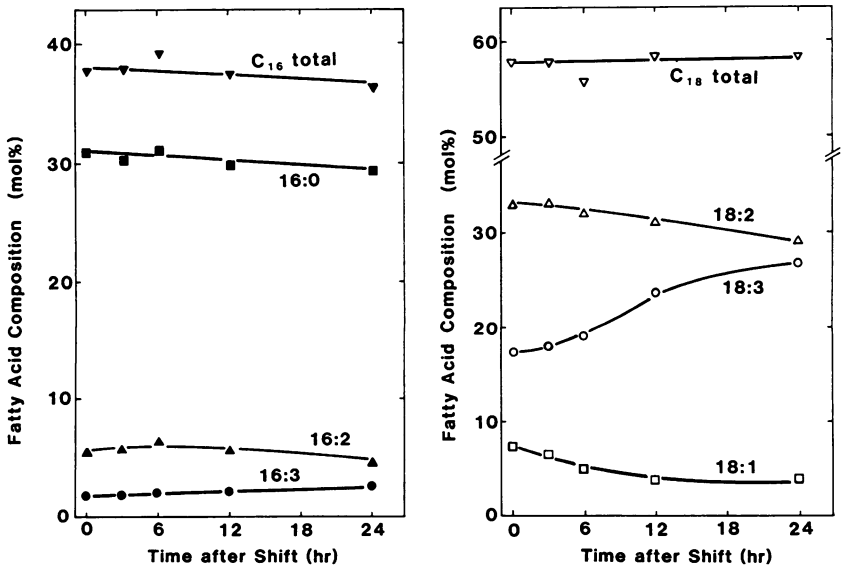

Figure 1. Changes in fatty acid composition after shift of CO_2-concentration from 2 to 0.04%.

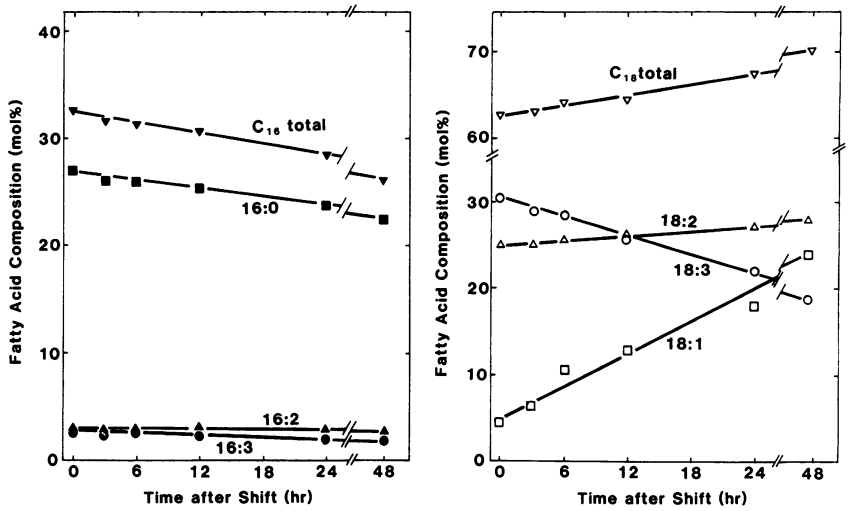

Figure 2. Changes in fatty acid composition after shift of CO_2-concentration from 0.04 to 2%.

unit weight of cells and the cellular growth. When low-CO_2 cells were transferred to high-CO_2 condition, both pcv and the total amounts of fatty acids increased. The increases of pcv and the total amounts of fatty acids were almost at the same rate, when the pcv at the transfer was low enough, *e.g.* 1 mL pcv/L (data not shown). In Figure 3, however, where the initial pcv was 2.7 ml pcv/L, the increase in the total amounts of fatty acids was greater than the increase in pcv. The increase of total fatty acids was due to the increases of 16:0, 18:1, and 18:2. The amount of 18:3, on the other hand, remained almost constant during 48 h. Thus the synthesis of 16:0, 18:1, and 18:2 was accelerated, but 18:3 was not decomposed during this period. Therefore, the changes in fatty acid composition shown in Figure 2 are brought about by the accelerated syntheses of 16:0, 18:1, and 18:2 and a dilution of the preexisting 18:3 with newly synthesized more saturated fatty acids.

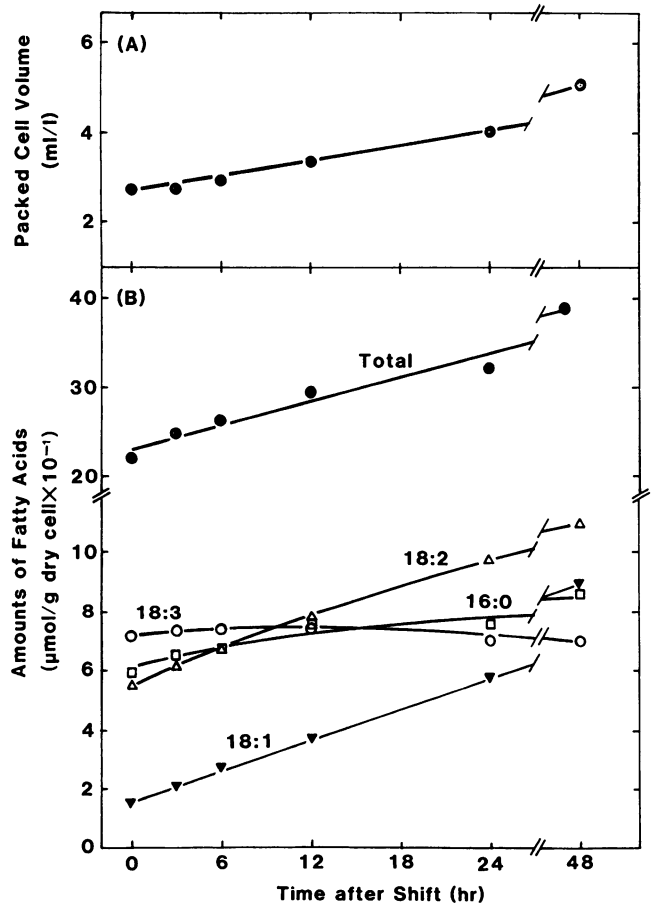

Figure 3. Changes in packed cell volume (A) and amounts of fatty acids (B) shift of CO_2-concentration from 0.04 to 2%.

Effect of CO_2 Concentration on Fatty Acid Composition in Various Algal Species

In two species of green algae (*C. reinhardtii* and *D. tertiolecta*), similar CO_2-dependent changes in the degree of unsaturation of fatty acids found in *C. vulgaris* 11h (Table IV) were also observed. But these changes were smaller than that of *C. vulgaris* 11h. Similar results were reported with *C. reinhardtii* by Sato (13). However, no difference in fatty acid composition was found between low- and high-CO_2 cells of two species of cyanobacteria, *Anabaena variabilis* and *Anacystis nidulans*, one species of red alga, *P. cruentum*, and *E. gracilis* (Table IV).

DISCUSSION

The fatty acid composition of *Chlorella vulgaris* 11h varied in response to CO_2 concentration given during growth. Desaturation was less when the cells were grown under high CO_2 condition. On the other hand, chain length of fatty acid was not influenced by CO_2 concentration. Of three species of green algae tested, the CO_2-dependent variations in fatty acid composition of *C. vulgaris* 11h was most variable. The greatest response to a downward shift of CO_2 concentration was the desaturation of 18:2. From an upward shift of CO_2 concentration, the response was represented by the decrease in unsaturation of fatty acids. Complex lipids are believed to be

Table IV. *Fatty Acid Composition of Various Algal Cells Grown with Air or Air Enriched with CO_2*

Fatty Acid	Fatty Acid Composition											
	Chlamydomonas reinhardtii		*Dunaliella tertiolecta*		*Porphyridium cruentum*		*Anabaena variabilis*		*Anacystis nidulans*		*Euglena gracilis*	
	Air	4%CO_2	Air	5%CO_2	Air	5%CO_2	Air	5%CO_2	Air	5%CO_2	Air	5%CO_2
						mol%						
14:0	3.0	1.5	0.8	1.4	ND	ND	3.4	4.8	4.0	2.9	ND	ND
14:1	ND[a]	ND	ND	ND	ND	ND	ND	ND	4.1	3.0	ND	ND
16:0	26.6	38.2	22.9	24.2	46.9	44.8	35.8	35.4	49.2	49.9	23.4	25.6
16:1	3.2	0.2	6.3	2.3	2.1	3.1	21.4	17.0	38.7	38.8	11.6	9.2
16:2	2.9	0.2	2.5	3.4	ND	ND	1.9	2.3	ND	ND	ND	ND
16:3	3.3	0.8	2.4	2.8	ND	ND	ND	ND	ND	ND	ND	ND
16:4	10.9	8.9	16.1	17.3	ND	ND	ND	ND	ND	ND	ND	ND
18:0	ND	ND	ND	ND	ND	0.7	ND	ND	ND	ND	ND	ND
18:1	16.7	18.7	2.8	5.0	ND	1.3	7.4	7.5	4.0	5.4	7.7	7.6
18:2	10.2	11.3	10.8	12.4	8.8	8.1	14.3	15.5	ND	ND	9.2	6.9
18:3 + 18:3 (n-6)	23.1	20.1	35.2	30.9	ND	0.5	16.0	17.6	ND	ND	24.6	30.8
20:2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.9	2.8
20:4	ND	ND	ND	ND	21.9	22.1	ND	ND	ND	ND	5.1	5.3
20:5	ND	ND	ND	ND	20.3	19.4	ND	ND	ND	ND	15.5	12.4
a.n.d.[b]	1.74	1.41	2.13	2.09	2.09	2.08	1.09	1.13	0.47	0.47	2.15	2.10

[a] ND = not detected. [b] Average number of double bonds per fatty acid molecule. The results are the average of two or three separate experiments.

substrates for the desaturation from 18:2 to 18:3 (15). The results presented in this paper imply that the activity of the lipid-linked desaturation might be affected by CO_2 concentration. With heterotrophically growing cells of *Chlorella fusca*, Dickson *et al.* (8) had reported that the amount of 18:1 on a dry weight increased when the CO_2 concentration was raised from 1 to 30%. Their results are consistent with the results shown in Figure 3B.

The present study emphasizes that the environmental CO_2 concentration affects on membrane lipid composition in addition to photosynthetic characteristics. Under low CO_2 concentration, *de novo* synthesis of carbonic anhydrase is induced and a pyrenoid with starch sheath is developed in various eukaryotic microalgae (12, 18). Carbon dioxide is much more soluble in fats than water and the adsorption is quick (11). Therefore, the change in the degree of unsaturation of the fatty acyl chain in the membranes is one of the reactions which occurs in response to the decrease of CO_2 concentration.

A CO_2 effect on fatty acid composition could not be observed in cyanobacteria as well as *Euglena* and *Porphyridium*. Cells of cyanobacteria can accumulate inorganic carbon inside the cells during photosynthesis (10), to compensate for the ambient low concentration of CO_2. Thus the concentration of dissolved inorganic carbon inside the cyanobacterial cells may be maintained constant irrespective of CO_2 concentration during the growth. The reason why the CO_2 effect was not observed in *Euglena* and *Porphyridium* is not clear at this moment.

The CO_2-dependent differences of 18:2/18:3 ratio were much greater in galactolipids than in phospholipids (Table III). Because MGDG and DGDG are typical lipids of thylakoid membranes, it is reasonable to assume that the CO_2 effect on fatty acid composition reflects changes in the thylakoid membranes. In this respect, the report which showed a change in distribution of excitation energy toward PSI in low-CO_2 cells of *Chlorella, Chlamydomonas* and *Dunaliella* (7) suggests the change in thylakoid status between low- and high-CO_2 cells. Detection of variations of the fatty acids in cell membrane would be difficult, although it is possible that CO_2 concentration specifically influences the fatty acid composition of plasma membrane.

The decrease in the unsaturation level of fatty acids is well known during the temperature shift in a wide range of living cells (*e.g.* see ref. 14). On the contrary, a CO_2 effect on fatty acid composition has been observed so far only in three green algae, but needs to be extended to other green alga. When grown heterotrophically, polyunsaturated fatty acid content decreased in eukaryotic algal cells (21). Because CO_2 concentration inside the cells may be higher under the heterotrophic condition due to respiration, the decrease of the desaturation level might be partly interpreted as a result of high-CO_2 concentration in these cells.

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